

FIGURE 1--Replacement of ERP with small molecule

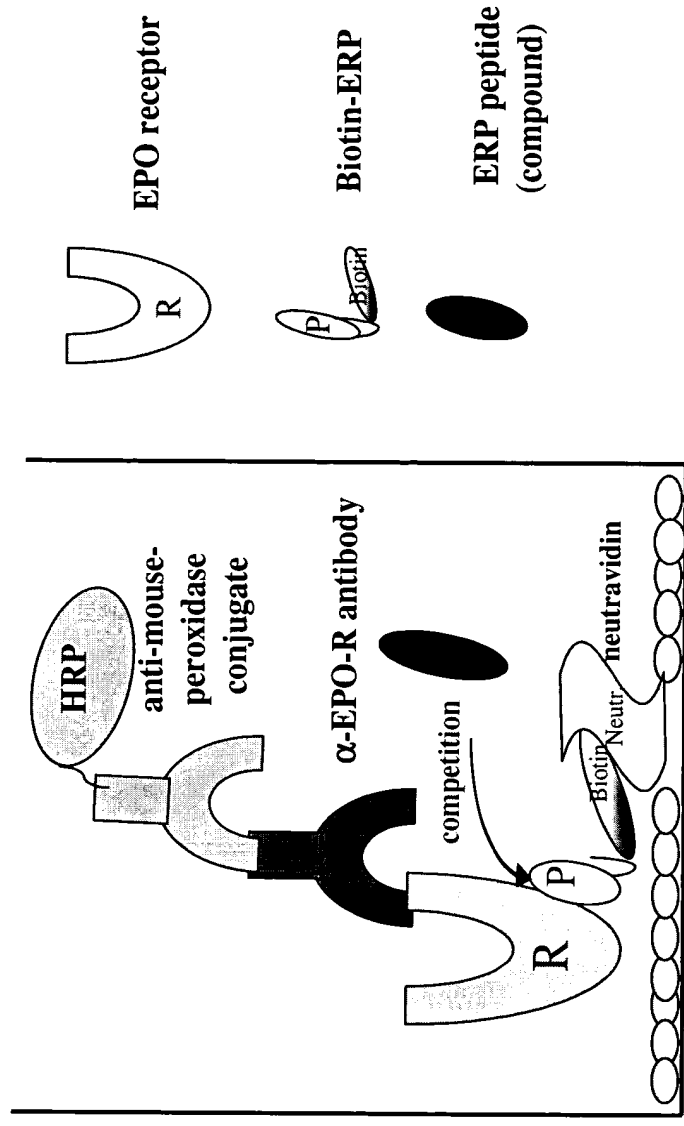
Primary screen

“peptide displacement assay”



Secondary screen

“functional assays”



## FIGURE 2--Role of small molecule in activation of

### EPO-R signaling pathway

- Study of small molecule effects and potential applications

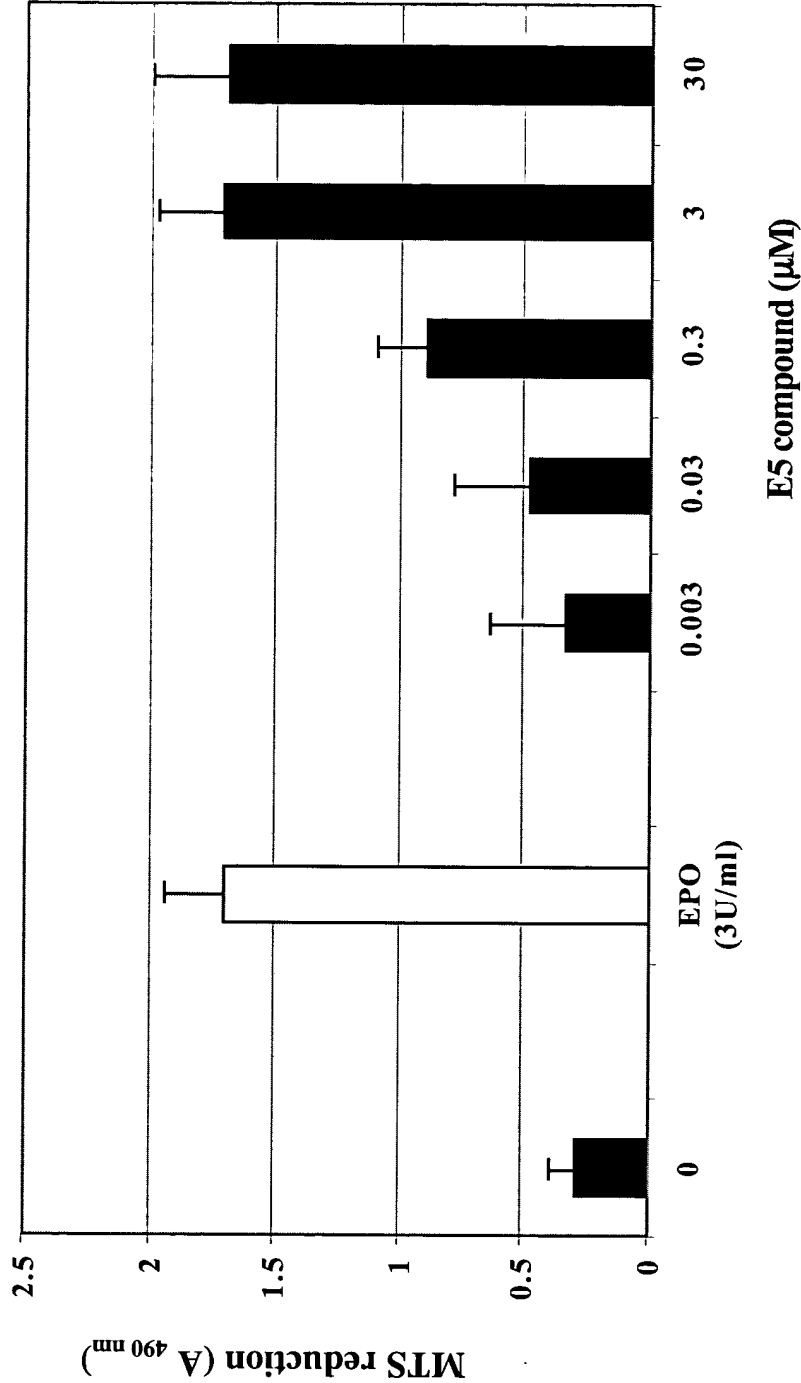
#### *In vitro studies*

- Erythroid system
  - Activation of proliferative and anti-apoptotic pathway(s)
  - Colony formation in fetal liver cells
  - CFU-e/BFU-e formation in bone marrow (mouse and human)
- CNS system
  - Activation of anti-apoptotic pathway
  - Survival of neural-like cells upon serum withdrawal

#### *In vivo studies*

- Effect in animals with carboplatin induced anemia; given I.p. and orally
- Reticulocytes levels in normal animals

FIGURE 3--Proliferative effect of E5 compound in  
TF-1 cells

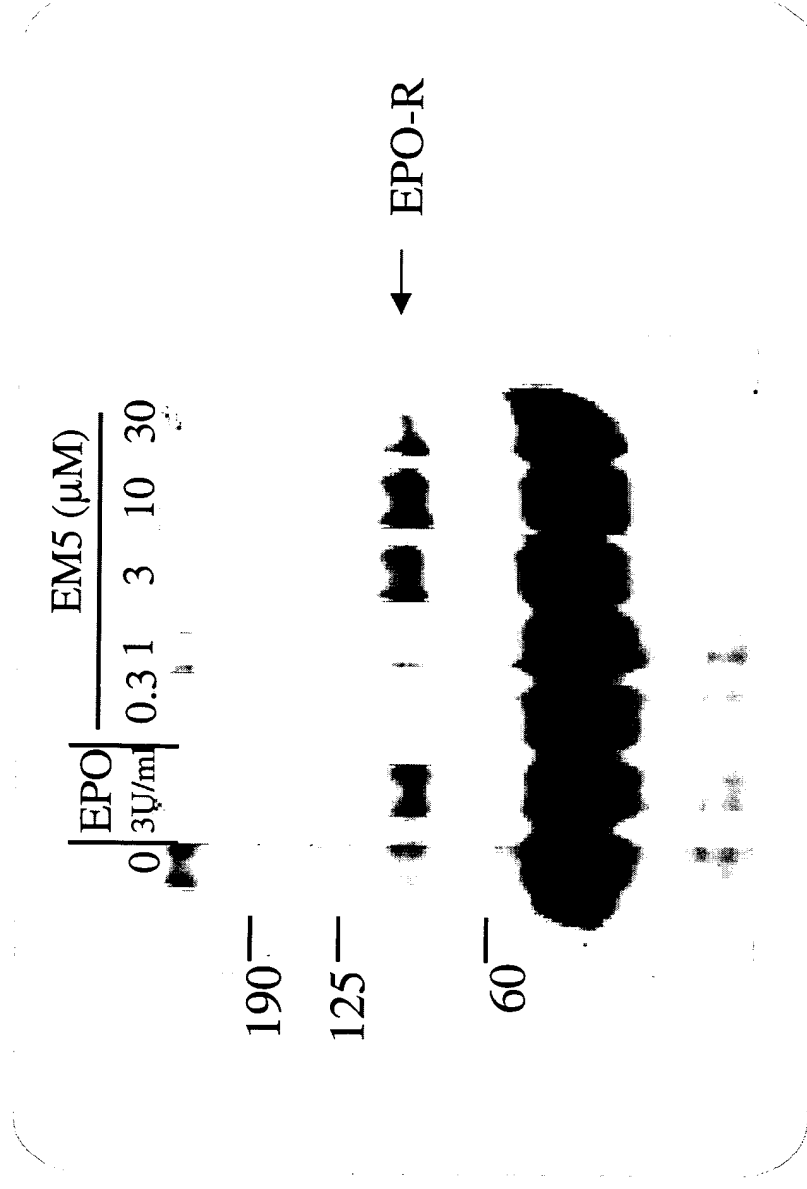


\* Same proliferative effect is observed with UT-7 cells  
\* No effect in FDCP1 cells

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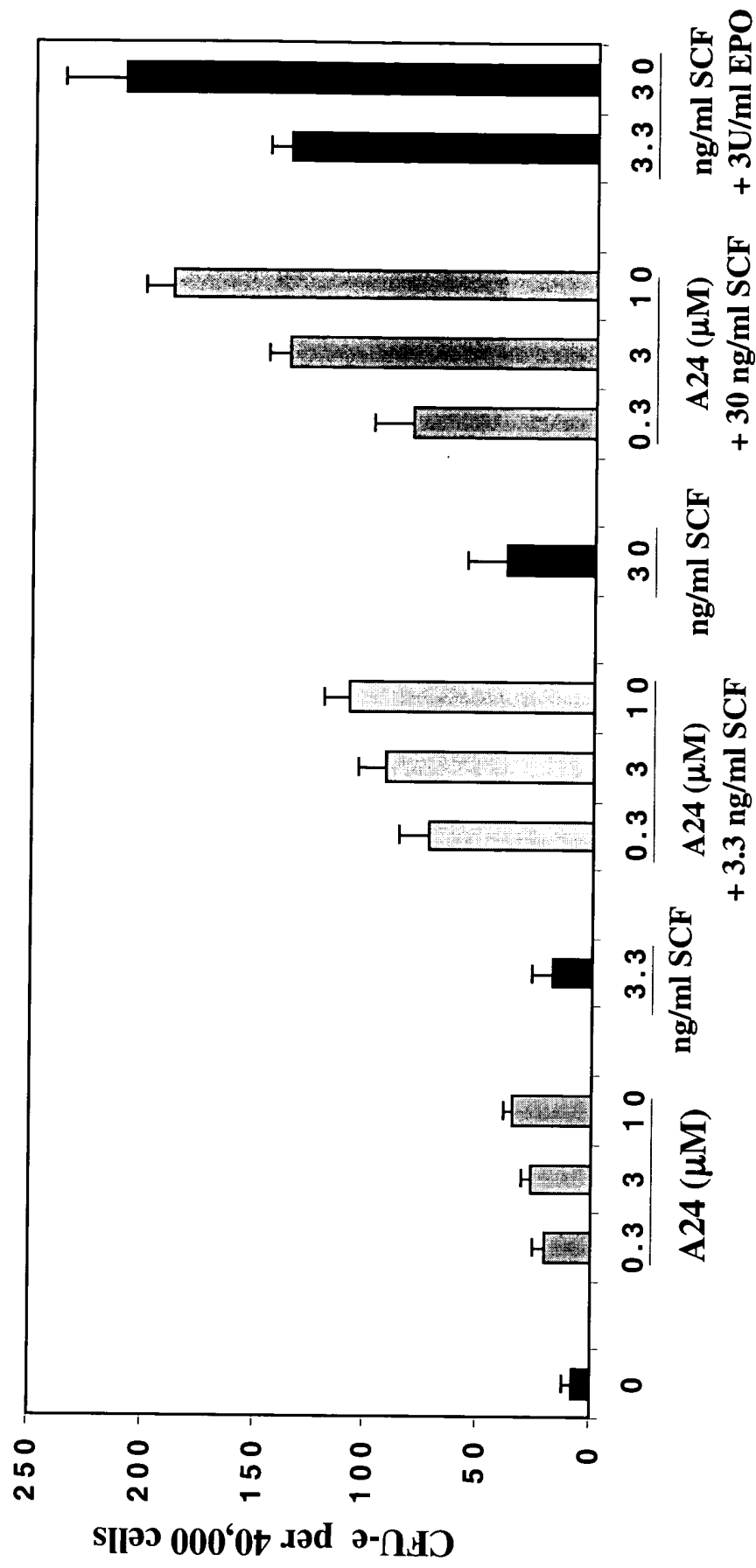
# FIGURE 4--Activation of signaling cascade through EPO-R by small molecule

- Small molecules bind to and activate/phosphorylate EPO-R (UT-7 cells)



IP: α-EPO-R Ab (Upstate technology)  
WB: α-PY Ab (Upstate technology)

FIGURE 5--Small molecules promote colony  
formation in the presence of SCF  
Fetal liver cells (day 3)



**FIGURE 6--Effect of EPO-like small molecule on erythroid colony formation in methylcellulose**  
**Human bone marrow**

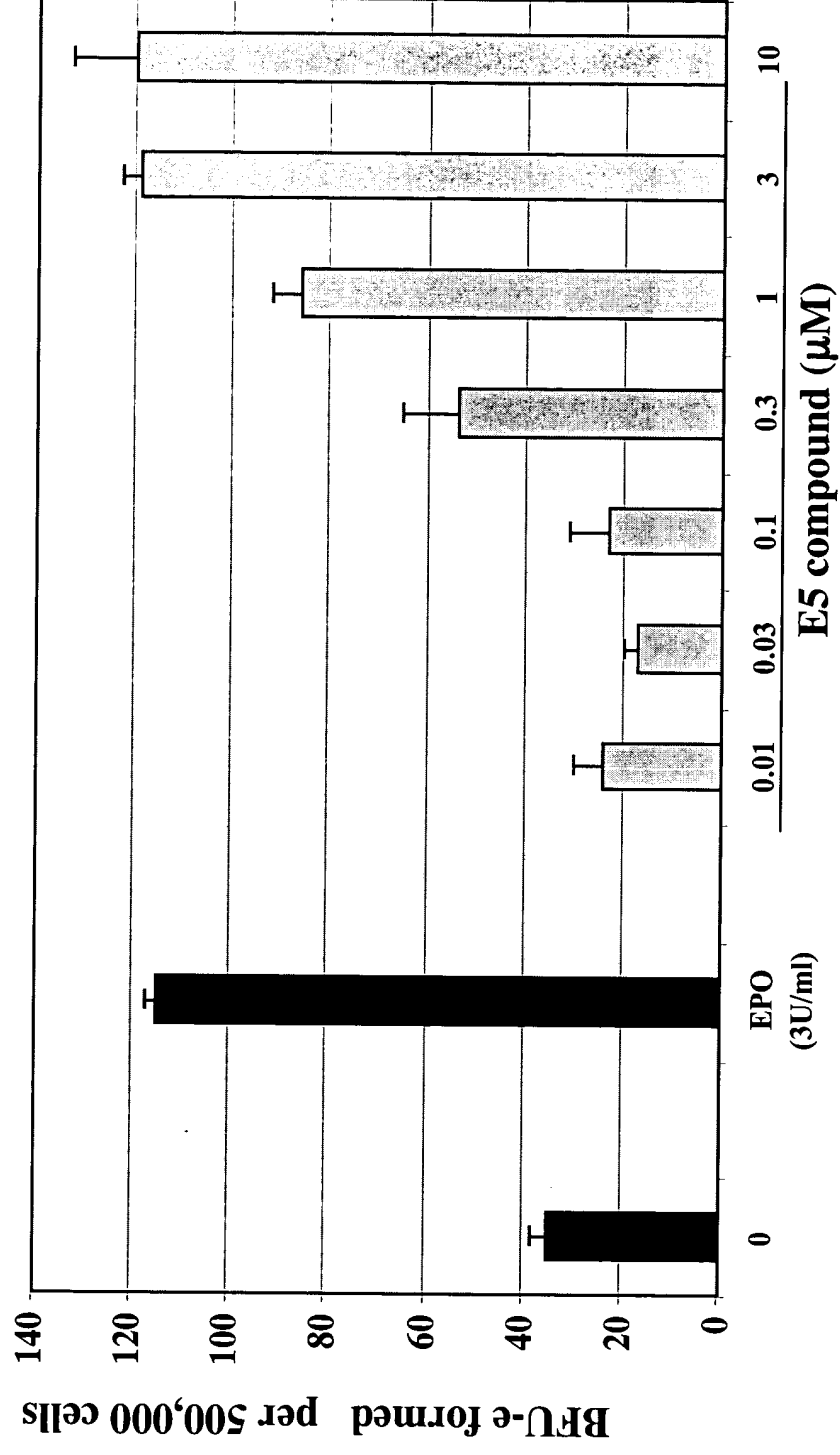
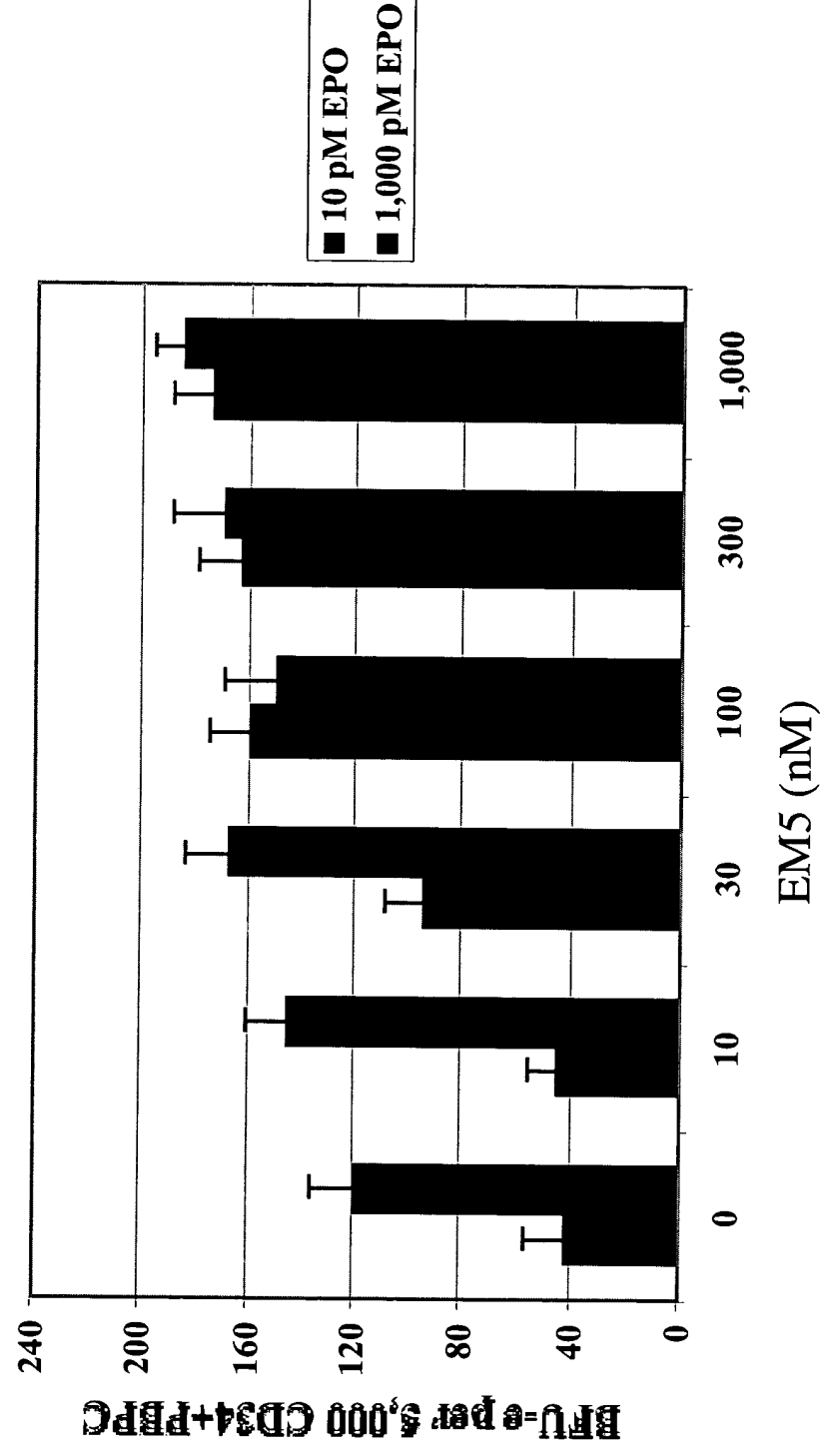
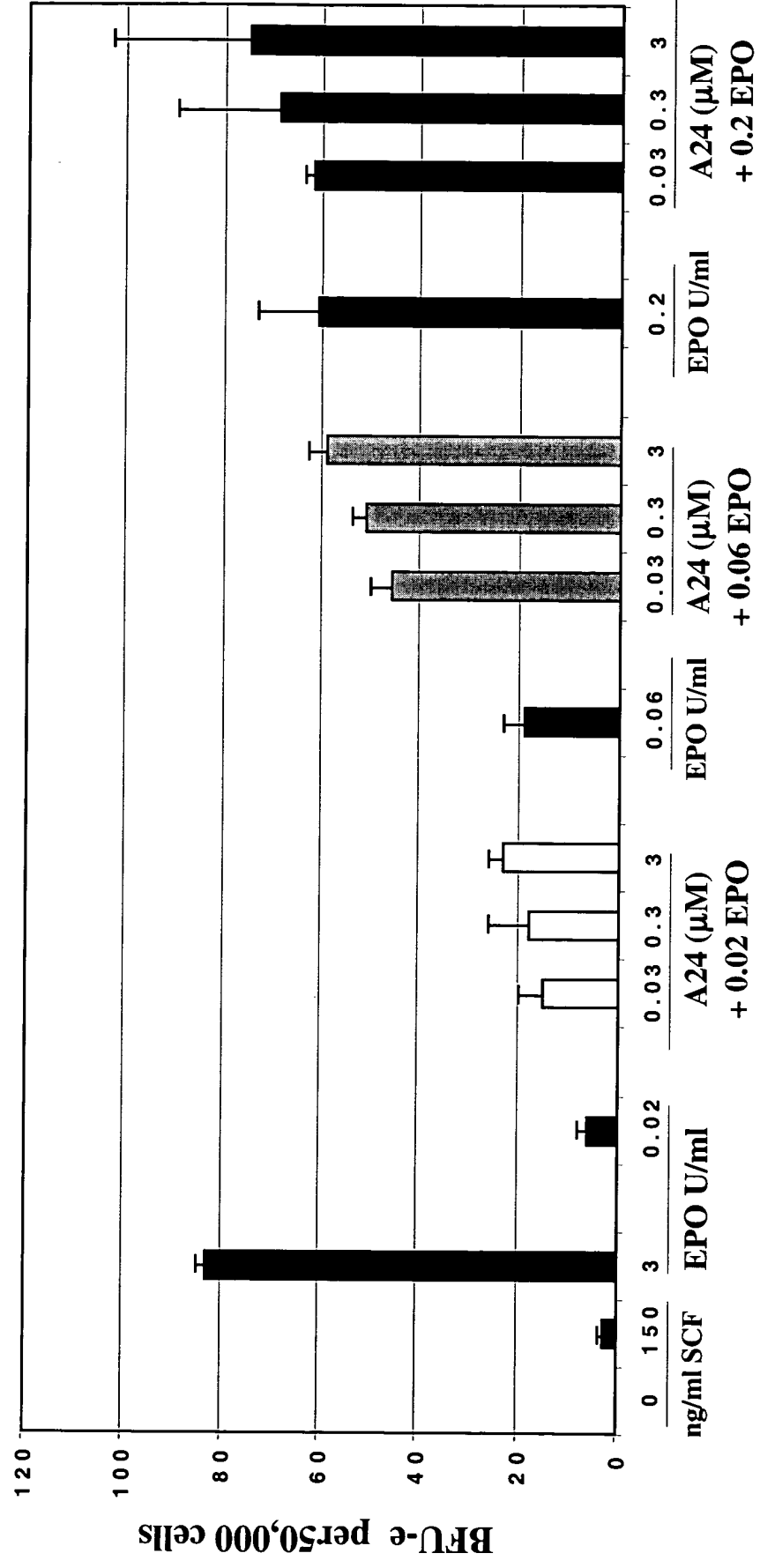


FIGURE 7-- Synergy between EM5 small molecule  
and EPO on erythroid colony formation  
Human bone marrow



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FIGURE 8 --Synergy between E5A24 small molecule and EPO on erythroid colony formation  
Human bone marrow (day 14)





# FIGURE 9

EFFECT OF EPO-LIKE  
ACTIVITY, SMALL  
MOLECULE - ON  
HEMATOCRIT IN 8  
WEEK OLD C57BL  
MALE MICE

Dose-response

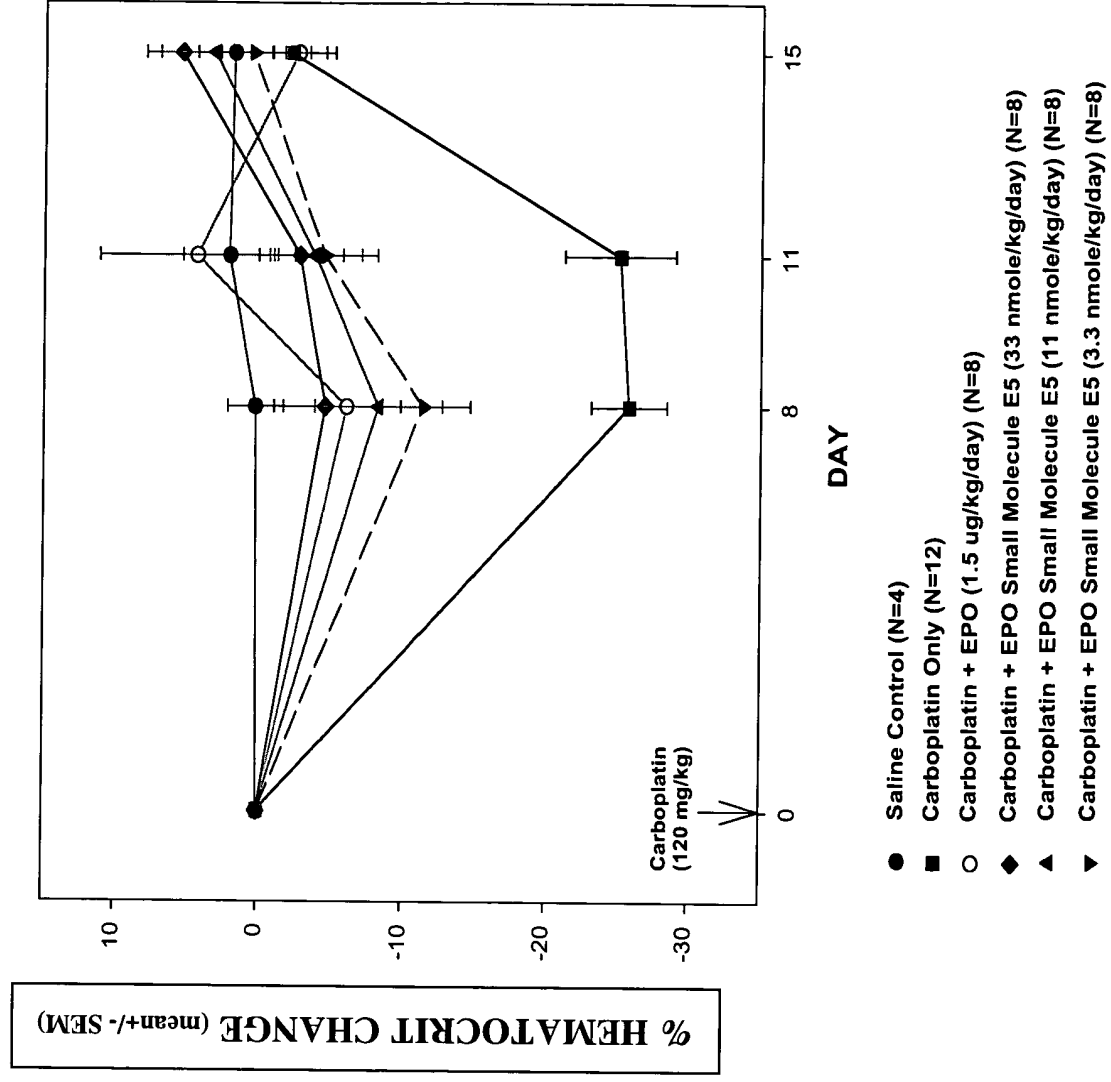
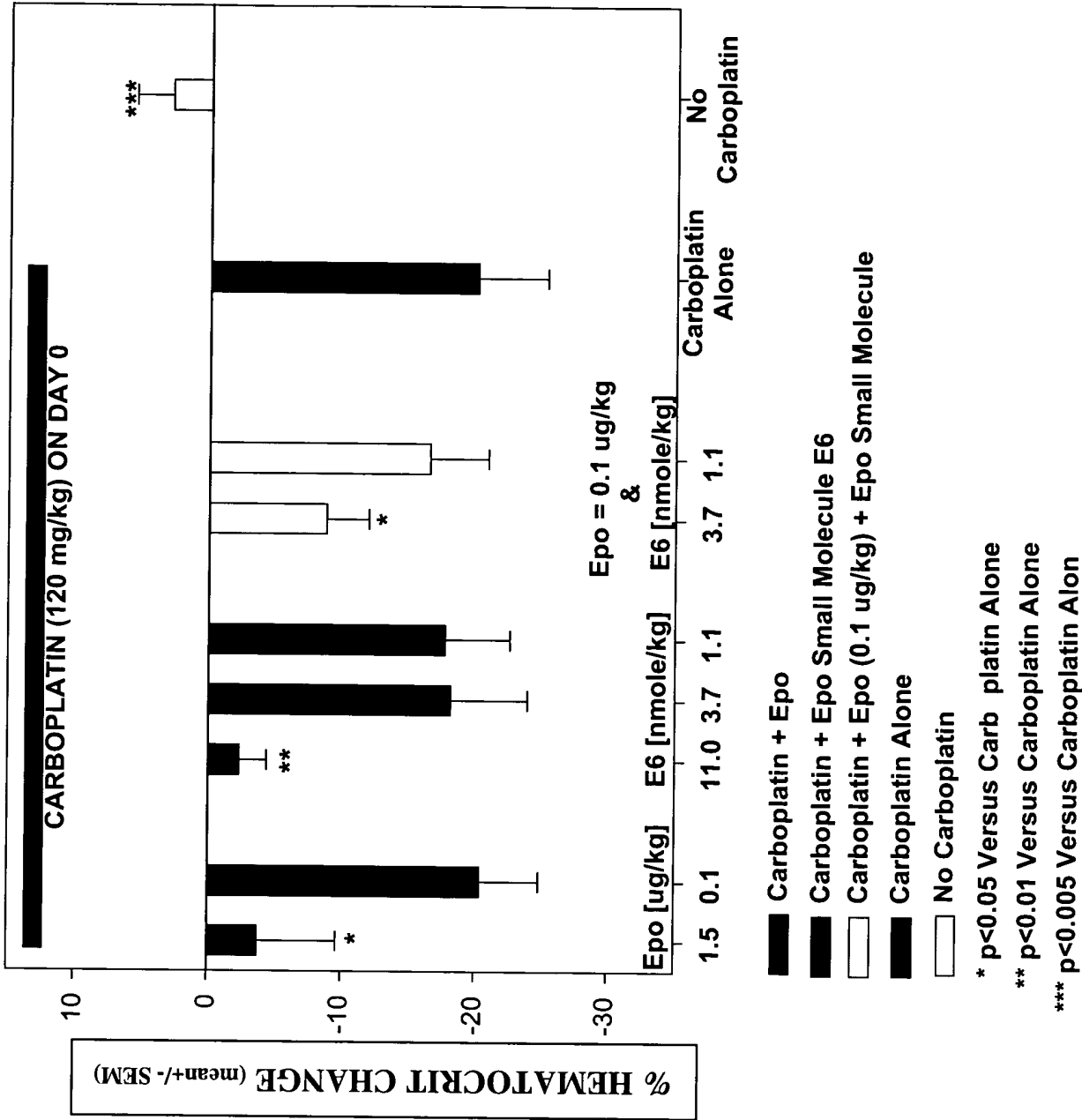


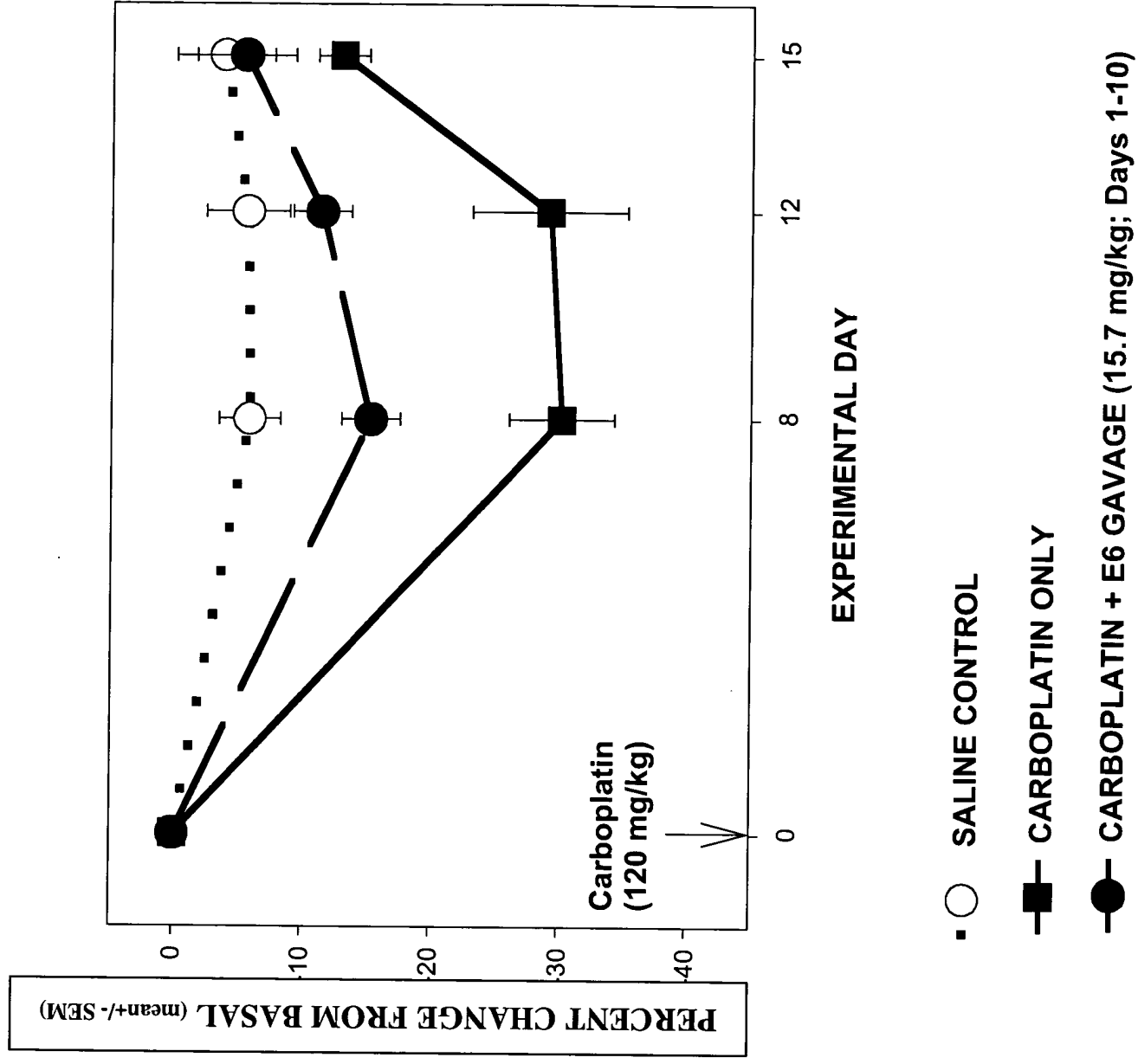
FIGURE 10

SYNERGISTIC  
EFFECT ON  
CARBOPLATIN-  
INDUCED ANEMIA BY  
ERYTHROPOIETIN  
WITH EPO SMALL  
MOLECULE E6 IN 8  
WEEK OLD MALE  
C57BL MICE  
DAY 10



**FIGURE 11**

**EFFECT OF E6  
ADMINISTERED  
BY GAVAGE ON  
HEMATOCRIT IN 8  
WEEK-OLD MALE  
C57BL/J MICE**



# FIGURE 12

Effect of EPO -  
small molecule  
on reticulocyte  
levels in normal  
animals

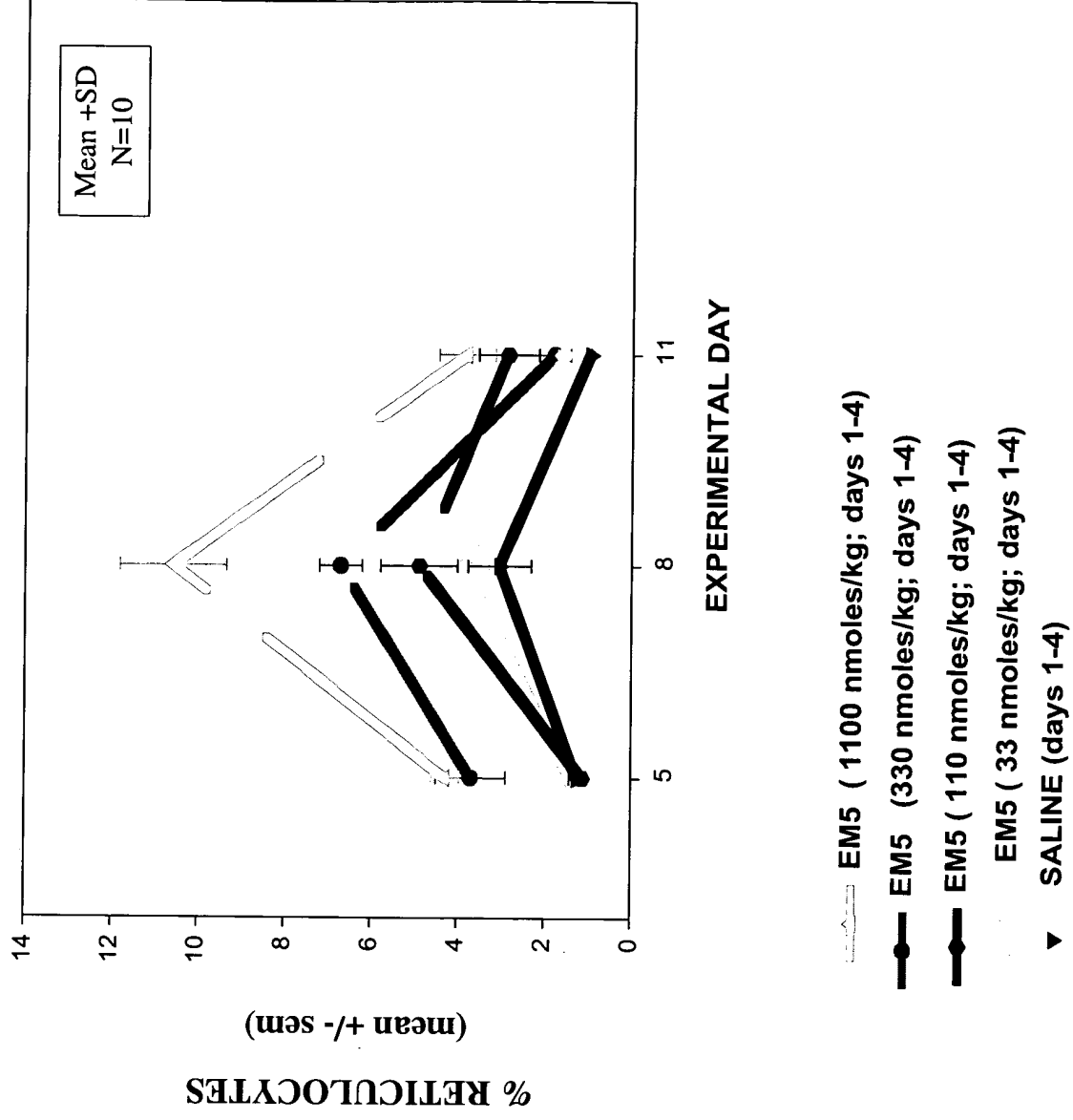
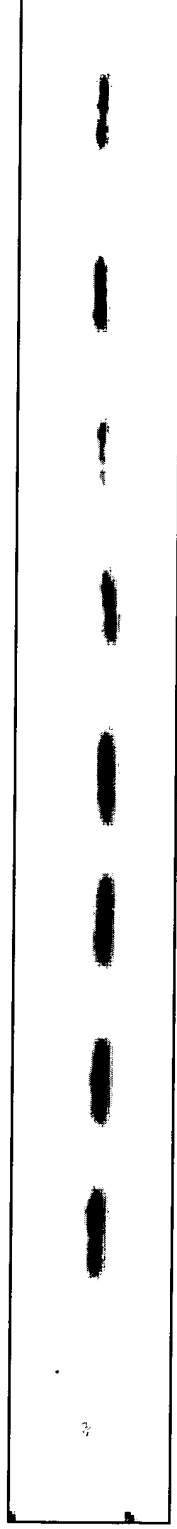


FIGURE 13 --Small molecule activates Bcl-X<sub>L</sub>  
expression

TF-1 cells

| Stimulation: | EPO (U/ml) |    |    |    | E5A24 (μM) |   |     | EM5 (μM) |    |   |
|--------------|------------|----|----|----|------------|---|-----|----------|----|---|
|              | 0          | 10 | 10 | 10 | 3          | 1 | 0.3 | 0.1      | 10 | 3 |



IP: anti Bcl-X<sub>S/L</sub>  
WB: anti Bcl- X<sub>L</sub>



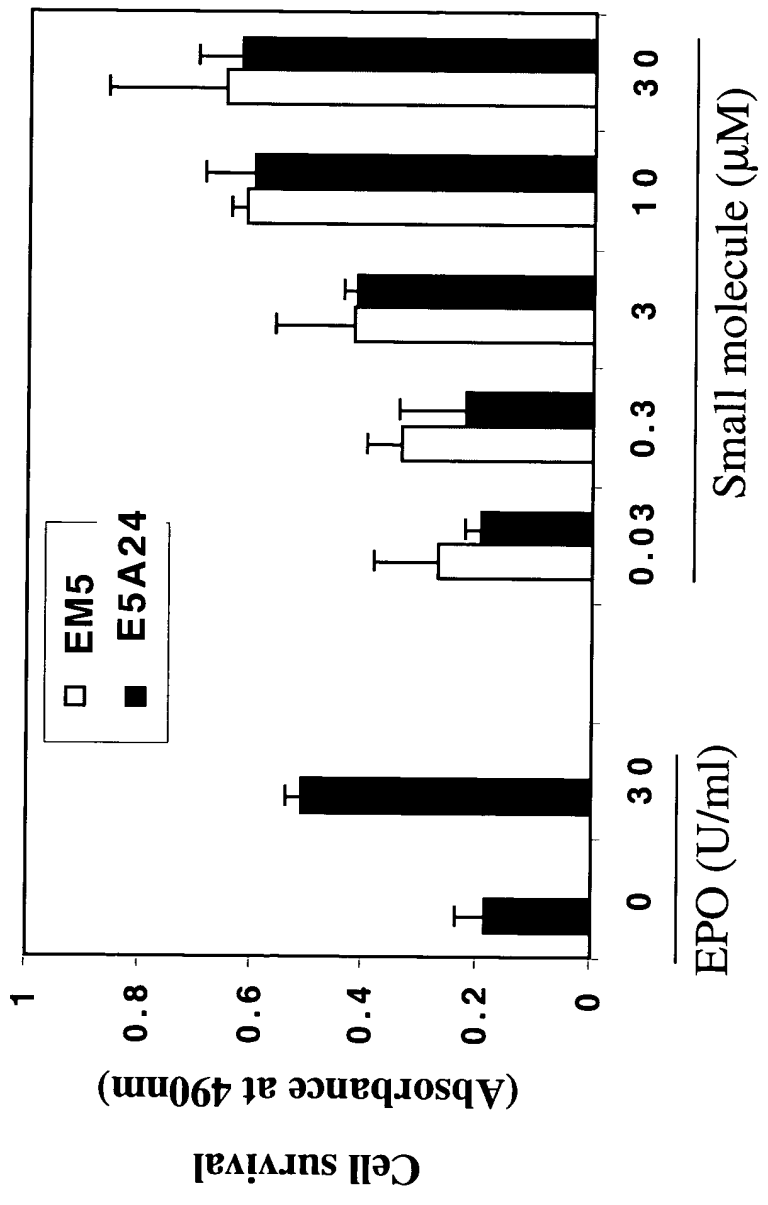
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# FIGURE 15 --Effect of small molecules on P19 cells

P-19 is a neural-like embryonal carcinoma cell line that undergoes apoptosis upon withdrawal of serum

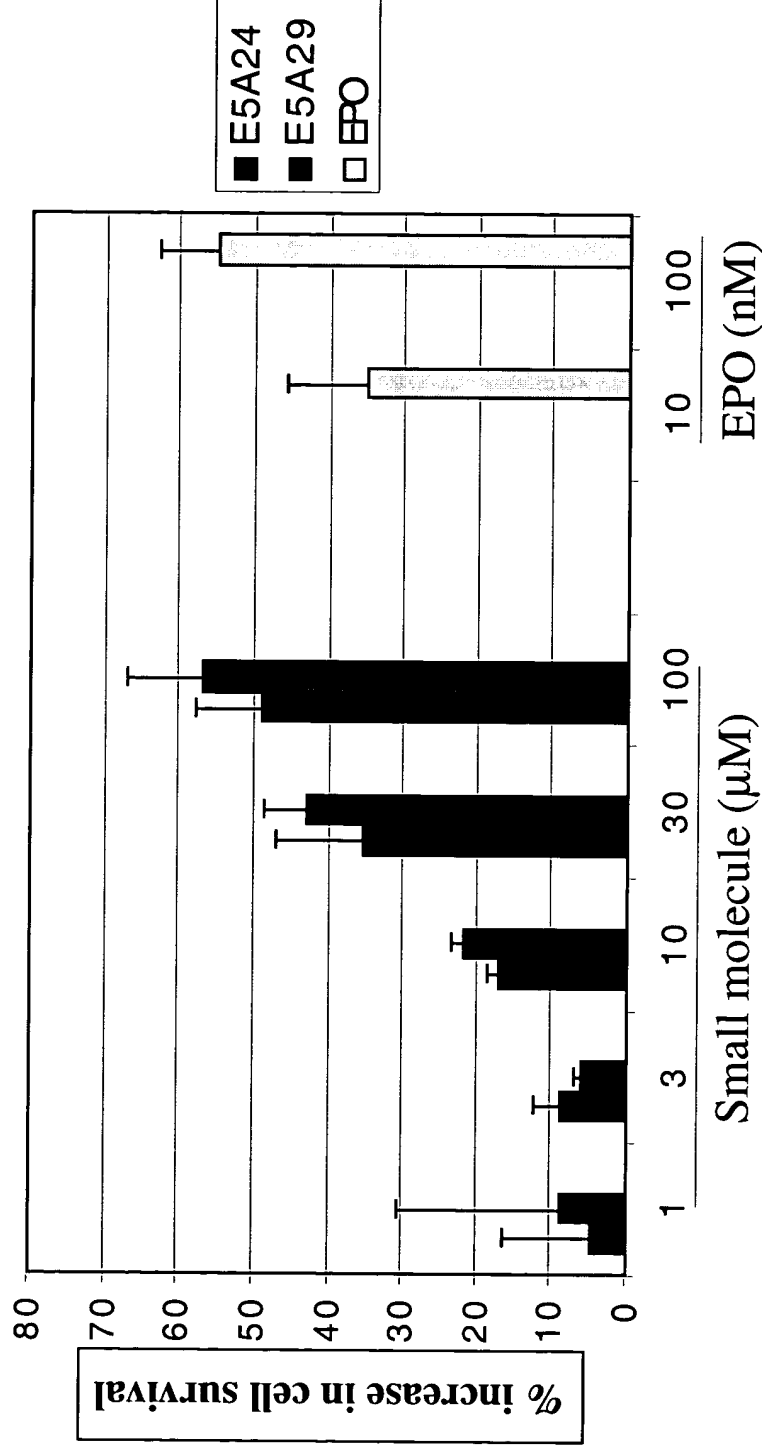
Protocol:

- \* Treatment with small molecule or EPO 24 hrs prior to serum withdrawal
- \* Serum withdrawal for 48 hours + EPO or small molecule



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FIGURE 16 --Small molecules prevent neuronal apoptosis  
after glutamate challenge



- \* Cortical Neurons isolated from Embryonic day 18 rats
- \* Treatment with small molecule or EPO for 24 hrs
- \* Challenge with 300 μM Glutamate for 24 hrs



## FIGURE 17 --Characteristics of EPO-like activity small molecules

- > Bind to EPO-R and Activate EPO-R signaling pathway in the presence and absence of hormone
  - Bind to different site than hormone and do not interfere with EPO binding
- > Act on early erythroid progenitors
  - CFU-e/BFU-e formation in fetal liver cells
  - Synergistic effect with EPO in Bone marrow cells (mouse and human)
- > Promote EPO like anti-apoptotic activity
  - Expression of Bcl-X<sub>L</sub> protein
  - Increase in neuronal survival during glutamate challenge
- > Restore hematocrit level in *in vivo* animal model, given I.P. and orally
  - Act in synergy with EPO
- > Increase reticulocyte levels in normal animals